J. Hortl. Sci.
Vol. 9(2):107-112, 2014

FOCUS

Production of doubled haploids in onion: A review

A.S. Dhatt and Prerna Thakur
Department of Vegetable Science
Punjab Agricultural University, Ludhiana -141 004, India
E-mail : ajmerdhatt@gmail.com

ABSTRACT

Onion suffers from high inbreeding depression and, as a result, inbreds that are developed lack genotypic and phenotypic uniformity. Gynogenesis has emerged as a potential strategy to address this drawback. Efforts have been made since the 1980s for identifying highly-responsive genotypes and for overall improvement of the protocol for bettering gynogenic frequency in onion. Besides improving media composition, identification of responsive explants and increasing the chromosome efficiency has remained a major area of focus over the years. This article purports to review progress made thus far in the induction of gynogenic haploids in onion, and challenges / opportunities associated with it.

Key words: Onion, *Allium cepa*, gynogenesis, haploid, chromosome doubling

INTRODUCTION

Hybrid cultivars of onion are considered to be superior to open-pollinated varieties due to higher uniformity and expressed heterosis. Onion populations possess deleterious recessive alleles, and due to inbreeding depression, breeding lines can be selfed for only up to two or three generations in this biennial crop. Thus, with conventional breeding it is difficult to obtain homozygous inbreds for complete genetic and phenotypic uniformity in the resultant hybrid. Doubled haploid (DH) production is an alternative strategy for complete homozygosity and phenotypic uniformity to obtain inbred lines in onion (Bohanec, 2002). Spontaneous development of haploid plants was first described in *Datura stramonium* (Blakeslee *et al*, 1922), followed by tobacco, wheat and several other species (Forster *et al*, 2007). *In situ* induction of maternal haploids has been possible by pollinating with pollen of the same species (maize), irradiated pollen (cucumber, melon, squash, watermelon, apple, mandarin, blackberry, European plum, sweet cherry, kiwifruit, pear, carnation, rose, petunia, sunflower and *Nicotiana*), pollen from a wild relative (barley, potato), or unrelated species (wheat). Induction of *in vitro* gynogenesis using un-pollinated flower parts has also been successful in several species like cucumber, squash, gerbera, sunflower, wheat, barley, onion and sugar beet (Murovec and Bohanec, 2012).

Haploidy has a great potential in onion breeding, as, hybrids are derived mainly from partial inbred lines. Partial inbred lines also require 14-16 years to be developed due to the biennial life cycle of the crop. Therefore, induction of di-haploids can greatly reduce the time and resources required for developing inbreds (Bohanec *et al*, 1995). The major factors affecting haploid induction include genotype, physiological condition of donor plants, developmental stage of the gametes (microspores, megaspore), pre-treatment, composition of culture medium, and physical factors during tissue culture (Murovec and Bohanec, 2012). Haploid plants may be obtained from male or female gametic cells. However, species differ in their ability to induce haploids *via* androgenesis or gynogenesis (Bohanec, 2002). It has been observed that response of anther to haploid induction is not successful in onion (Keller and Korzum, 1996). Haploid induction *via* un-pollinated flowers or ovaries is under practice for the last 20 years, after the first discovery by Muren (1989). A high rate of success in onion through gynogenesis was observed by Bohanec *et al* (1995), Luthar and Bohanec (1999) and Bohanec (2009). Induction of maternal haploids, called gynogenesis, can be achieved with

in vitro culture of various parts from un-pollinated flower such as ovules, placenta with ovules attached, ovaries, or whole flower-buds as discussed hereunder:

Choice of explant, developmental stage and sterilization-protocol

In onion, a large number of anther culture experiments failed to generate haploids (Keller and Korzun, 1996). However, gynogenic haploid induction could be achieved through culture of un-pollinated ovules/ ovaries/ whole flower-buds (Bohanec, 2002). Keller (1990) observed that ovule culture was the most laborious and yielded the lowest number of embryo regenerants. Therefore, this is no longer used for haploid induction in onion (Bohanec, 2002). Flower bud culture is the simplest way of inducing gynogenic haploids in onion and has been used in many recent studies by various workers. Bohanec (2002) and Bohanec and Jakse (1999) estimated that extraction of ovaries from pre-cultured flower buds *vis-a-vis* whole-flower culture required more labour, while, gynogenic response of ovary *vis-a-vis* flower culture was often similar. Also, whole-flower culture was found to have the disadvantage of growth of basal callus (not so in ovary cultures), resulting in production of low-quality haploid embryos. Flower culture has been reported to show a possibility of somatic regeneration from callus, though this was genotype-dependent. Currently, the most efficient method is to plate whole onion flowers, without sub-culture, to induce haploid plants from cells of the female gametophyte (Bohanec *et al*, 2003). According to Muren (1989), flower buds 3-5 days prior to anthesis were superior to either older or younger ones. Michalik *et al* (2000) concluded that small young buds of 2.8-3mm length produced significantly fewer embryos than older ones of 3.5-4.5mm length, while displaying genotype specificity. Alan *et al* (2004) collected unopened flowers of all sizes (2-5mm) and separated them into three size groups, viz., small (<2-2.5mm), medium (2.25-4.5mm) and large (>4.5mm) before culturing them in plates. Small flower buds responded poorly, whereas medium-sized buds gave the best results. Musial *et al* (2001) reported that in onion, small and large flowers containing megaspore mother cells and mature embryo sacs, respectively, were less responsive than medium-sized flowers having 2-4 nucleate embryo sacs.

Before placing in culture, flowers were surface-sterilized with 96% ethanol for 1 min, followed by treatment with 10% sodium hypochlorite (60g/dm^3 active chlorine) containing a few drops of Tween 20 for 15 min, and then rinsed thrice in sterilized water (Ponce *et al*, 2006). Bohanec *et al* (1995) and Jakse *et al* (1996) reported dissecting the flowers, followed by sterilization for 10 min in 5% (v/v) solution of sodium hypochlorite with a few drops of Tween 20 added in. Rinsing in sterile water followed this, after which the largest unopened flowers were selected and inoculated. Geoffriau *et al* (1997) reported washing of the excised umbel in 96% ethanol for 1 min, sterilization in 0.5% sodium hypochlorite solution for 15 min, and rinsing thrice in sterilized distilled water.

Pre-treatment

The principal benefits of optimizing pre-treatment for stock plants was to eliminate variation arising from external factors, thus maximizing gynogenic responsiveness in cultured flower-buds of onion across its flowering season. Pre-treating the stock plant has been shown to significantly affect frequency of gynogenic embryogenesis from whole flower-bud cultures in a range of onion genotypes (Puddephat, 1999). Stress treatment is the most common factor affecting embryogenesis, where cold or heat shock, or starvation treatments are commonly used. Without imposing stress, a change from gametophytic to the sporophytic phase is very difficult. Pre-treatments can be applied to different types of explants, with varying severity and duration, resulting in different regeneration efficiencies as well (Chen *et al*, 2010). Puddephat *et al* (1999) observed that pre-conditioning of stock ovaries significantly influenced gynogenic embryogenesis. Also, high illumination was found to be beneficial in onion. Flower buds excised from stock plants maintained at 15ºC were ten times more responsive than those taken from plants raised under glasshouse conditions, or held at 10ºC. It has been established that lowering donor-plant growth temperature in the final phase/s of flower development improves the efficiency of gynogenesis in edible onion. Alan *et al* (2004) also reported that flower buds (3-5mm) from stalks of plants stored at 10ºC remained responsive to induction of gynogenesis and were comparable to fresh, large flower-buds. Bohanec *et al* (1995) reported the use of Parafilm for sealing *petri* dishes and exposed them to 16-hr light/8-hr dark photoperiod at an illumination of 78µE m^{-2}s^{-1} at 25±1ºC. Jakse *et al* (1996) exposed sealed *petri* dishes to 16/8hr photoperiod at 23-25 ºC and illumination of 78 µmol m^{-2}s^{-1}. Geoffriau *et al* (1997) cultured flowers at 20ºC night and 22ºC day temperature under a 16h photoperiod of 100 µmol/s/m^2 photosynthetically active radiation- PAR (400-700 nm) supplied by fluorescent

tubes (58W). Bohanec and Jakse (1999) exposed Parafilm-sealed *petri* dishes to 16/8 hr photoperiod at 21-23ºC and 80µmol $m^{-2}s^{-1}$ illumination.

Media composition

Basal mineral composition: The three most-often used combinations of macro- and micro-elements have been reported as B_5 (Gamborg *et al*, 1968), BDS (Dunstan and Short, 1977) and MS (Murashige and Skoog, 1962). Chen *et al* (2010) mentioned that organic-nitrogen source, carbohydrates and growth regulators are the most-often modified components. Generally, gynogenesis spans two or more stages, and each stage may have distinct nutritional requirements. During induction, ovaries require low levels of growth regulators and are then transferred to a medium with higher concentrations of growth regulators. Ponce *et al* (2006) compared regeneration of gynogenic embryos at gellan-gum concentrations of 7g/dm^3 and 3 g/dm^3, and, the higher concentration was found to be more effective. Bohanec *et al* (1995) tested a 2-step culture procedure for generating gynogenic plants using flower pre-culture, followed by ovule or ovary culture method of Campion and Alloni (1990). Further, Bohanec and Jakse (1999) found a single-step culture less time-consuming and three times more efficient than the two-step ovary culture. Alan *et al* (2004) compared the single-step culture (BDS medium) and two-step culture (BDS/B_1 medium) for induction of gynogenic plants, and concluded that, single-step strategy was more convenient for large-scale induction of haploids.

Use of polyamines: Polyamines have been reported as essential for growth and development of living tissues, by Ponce *et al* (2006). Martinez *et al* (2000) showed that putrescine and spermidine induced the onset of embryogenesis in onion and enhanced the number of gynogenic embryos / plantlets obtained. Embryo induction was greatest with a combined treatment of 2mM putrescine and 0.1mM spermidine. Addition of putrescine alone did not give any significant effect, whereas, use of spermidine after 15 days of culture promoted further embryo maturation and plantlet formation. Ebrahimi and Zamani (2009) reported production of highest number of gynogenic embryos in media supplemented with 0.01 mM BA, 0.01 mM 2,4-D, 2mM putrescine and 0.1mM spermidine in onion flower buds. Ponce *et al* (2006) found putrescine to be inhibitory at high concentration 0.16g/dm^3 in all the genotypes studied. CCC (2-chloroethyltrimethyl ammonium chloride) at a concentration 0.1 g/dm^3 was found to increase gynogenic embryo production rate more than thrice when compared to Control. Genotype-specificity of medium influencing production rate of embryos was also observed. Various doses of growth regulators in different media combinations required for successful gynogenesis are tabulated below:

Growth regulator/s used	**Concentration in Induction Medium (mg/l)**	**Reference/s**
2,4-D + BAP	2+2	Muren,1989; Campion *et al*, 1992; Bohanec *et al*, 1995; Jakse *et al*,1996; Geoffriau *et al*, 1997
IBA + BAP	2.03+1.25	Keller, 1990
TIBA+ABA	0.2+1	Campion and Alloni, 1990
2,4-D+BAP; NAA+BAP	2+0.12,1+0.12	Campion *et al*, 1992
PAA+BAP	100+2	Jakse*et al*, 1996
2,4-D+BAP	2+2	Jakse*et al*, 2010; Bohanec and Jakse, 1999
2,4D+BA+ Putrescine+ Spermidine	0.01mM+ 0.01mM+ 2mM+0.1mM	Ebrahimi and Zamani, 2009
BA+2,4-D	2+2	Puddephat, 1999
Growth regulator/s	**Concentration in Regeneration Medium (mg/l)**	**Reference/s**
2iP+ IAA, 2iP+ NAA	2+1.5, 2+1	Puddephat, 1999
IBA	1	Muren, 1989
NAA+2,4D+ IAA+BAP+2iP	1+0.4+1,5+2+2	Campion and Alloni, 1990
TDZ	2, 2	Bohanec *et al*, 1995; Jakse *et al*, 1996
NAA + GA_3; NAA + BAP	1+1,1+2	Campion and Alloni, 1990
NAA+ 2iP	1+2	Campion and Alloni, 1990; Bohanec *et al*, 1995
IAA+BAP; IAA+2iP; IAA+GA_3; 2,4D+BAP; 2,4D +2iP; NAA+2iP+ ABA;NAA+ BAP+GA_3; BAP+GA_3+TIBA	1,5+2; 1,5+2; 1,5+1; 0,4+2; 0,4+2;1+2+1; 1+2+1;2+1+0.2	Campion and Alloni, 1990
IBA+BAP+GA_3	2+0,1,2+3,5	Keller, 1990; Campion *et al*, 1992

Effect of genotype

Genetic make-up of donor onion plant and growth conditions play the most important roles to succeed at gynogenesis. Theoretically, for haploid induction in onion, a maximum of 600% frequency can be expected. However, in practice, yields are low (Bohanec *et al*, 2001). Geoffriau *et al* (1997) tested variable genetic material from different regions across the world. They found that only two out of

18 onion cultivars showed a high gynogenic potential. In a similar study conducted by Bohanec and Jakse (1999), accessions from Europe, Japan and North America were analyzed, and they found the highest yield in North American cultivars. Very high variability was found among cultivars, and even within inbred lines. Michalik *et al* (2000) reported a maximum of 10% embryo yield in a breeding line out of 11 Polish cultivars and 19 breeding lines studied. Bohanec and Jakse (1999) demonstrated that, crossing responsive with non-responsive onion lines, resulted in increased gynogenic ability in the hybrid progeny. Jakse *et al* (2010) proposed an integrated method, wherein, the final proportion of haploid donors that regenerated DH plantlets doubled at the least, and reached up to 80%. Geoffriau *et al* (1997) reported that among genetic structures, inbreds regenerated significantly better than synthetics. Regenerants from inbreds were the most vigorous, whereas, synthetics were confirmed to be good donor material for quality embryos.

Determination of ploidy level and homozygosity

Different methods have been used for analyzing ploidy level. Chromosome count was performed on root tips (Muren, 1989; Campion and Alloni, 1990; Keller, 1990; Bohanec *et al*, 1995; Campion *et al*, 1995) or shoot tip cells (Campion *et al*, 1995). Bohanec *et al* (1995) performed chromosome analysis of root tips by staining *in vitro* grown hydrolyzed (70ºC, 3 min) root tip cells with acetocarmine (arrested in metaphase with 45mg/ 100ml 8-hydroxyquinoline). Martinez *et al* (2000) and Ebrahimi and Zamani (2009) determined chromosome number in root tip cells obtained from plantlets after treatmentw thn 0.1% colchicine for 3h, fixed in 3:1 ethanol: glacial acetic acid, digested in 1N HCl at 60ºC for 8 min and squashing in 45% acetic acid. For microscopic inspection of the karyotype, root tips were stained with 1% haematoxiline or 2% acetocarmine. Flow cytometry using leaf tissue has been a predominant method (Cohat, 1994; Bohanec *et al*, 1995; Campion *et al*, 1995; Jakse *et al*, 1996; Geoffriau *et al*, 1997; Javornick *et al*, 1998; Bohanec and Jakse, 1999; Alan, 2004). A protocol for flow cytometry was developed by Bohanec *et al* (1995) and the samples were analyzed on a FAC-Sort flow cytometer. Bohanec and Jakse (1999) used this method to analyze ploidy level of regenerants. To release the nuclei, leaves were chopped with a razor blade in 1 ml 0.1M citric acid containing 0.5% Tween 20, and the suspension was filtered through 50µm nylon gauze filter. A three-fold volume of dye solution containing 5.25µg/ ml 4,6-diamidino-2-phenylindole in 0.4M disodium hydrogen phosphate was added to the filtered suspension. Flow cytometry measurements were performed with Partec PAS-Iii flow cytometer equipped with a 100W high-pressure mercury lamp. Partec Ploidy Analyzer was also used for ploidy analysis (Anon., 2007).

In diploid species, spontaneous diploids are very useful in plant breeding, as, these are more stable than dihaploid generation through haploids treated with colchicines. However, homozygosity of the regenerants needs to be proved (Geoffriau *et al*, 1997). Jakse *et al* (1996) submitted regenerated plantlets to isozyme electrophoretic analysis to establish the frequency of homozygous / heterozygous origin. Bohanec *et al* (1995) used a modified electrophoresis technique, where, approximately 200mg fresh weight of leaf tissue (young leaves sprouted from donor plant bulbs, or, *in vitro* grown leaves) was crushed in 200µl of extraction buffer consisting of 15% (w/v) sucrose, 50mM Tris-HCl (pH 7.1) in 0.5% (v/v) Triton X-100. The extract was then centrifuged at 26500*x* g for 5 min at 4ºC; the supernatant was immediately loaded onto the gel. Stacking gel (2cm) consisted of 24.6 g/l acrylamide and 6.15g/l bisacrylamide, 4.0g/l Triton X-100, 0.7g/l ammonium persulphate, 0.6ml/l TEMED in 0.07M Tris (pH 7.8); and, the resolving gel consisted of 57.8g/l acrylamide and 2.2g/l bisacrylamide, 0.37g/l ammonium persulphate, 0.37ml/l TEMED, 2.0G/L Triton X-100 in 0.07M Tris (pH 7.8). As electrode buffer, 1g/l Tris and 5.52 g/l barbitone (pH 7.3) was used. Electrophoresis was carried out at 10ºC for 3h at a constant voltage of 225 V. This method was used again by Bohanec and Jakse (1999), who had used another method of RAPD analysis earlier (Bohanec *et al*, 1995).

Genome-doubling procedures

The spontaneous doubling of gynogenic plants is a rare event in the bulb-onion (Bohanec, 2002). The major problem in genome doubling in onion is inaccessibility of the apical meristem of adult field-grown plants. Therefore, chromosome doubling of haploid onion plantlets should be attempted during *in vitro* propagation. Jakse *et al* (2003) mentioned that an efficient method for chromosome doubling should take into account survival rate and chromosome-doubling efficiency. Alan *et al* (2004) studied various parameters in chromosome doubling experiments with 100-400mg/l dose of colchicine, in liquid and solid media, for 24 and 48 hours. For high rate of recovery of diploids, exposure of basal explants from 2-4 month old *in vitro* haploid plants to 200-400 mg/l colchicine in liquid medium for 48h was

suggested, wherein, 10% survival of explants with APM (Amiprofos-methyl) treatment was reported. Geoffriau *et al* (1997) compared the efficiency of colchicine and oryzalin, and the best results were obtained with either 2.5mM colchicine (up to 65.7% diploids) or 50µM oryzalin (up to 57.1% diploids). Both the chemicals induced mixoploids and affected plant regeneration, but better plant-quality was obtained with oryzalin. Grzebelus *et al* (2004) reported oryzalin, trifluralin and APM as better agents than colchicine for *in vitro* chromosome doubling in onion tissue. However, APM is recommended due to its low toxicity. Bohanec and Jakse (1997) tested the effect of oryzalin and colchicine on halved basal shoots. Diploidization with oryzalin (67%) was better than with colchicine (21%). Jakse *et al* (2003) reported that the 2-day treatment in liquid media supplemented with 50µm APM gave the highest percentage of diploids (36.7%), but the survival rate was reduced to 52.5% that in the non-treated control. Alan *et al* (2007) compared the efficiencies of three antimitotic agents (APM, colchicine and oryzalin) and recommended APM (100 or 150µM) due to its low toxicity and ability to yield results comparable to that with colchicine (750 or 1000µM).

Thus, it can be concluded that complete homozygosity through DH approach can be attained in less time than traditional breeding approaches. However, genotype-specific protocols for induction of haploidy and chromosome doubling are required for success in onion.

REFERENCES

Alan, A.R., Brants, A., Cobb, E.D., Goldschmied, P.A., Mutschler, M.A. and Earle, E.D. 2004. Fecund gynogenic lines from onion (*Allium cepa* L.) breeding materials. *Pl. Sci.,***167**:1055-66

Alan, A.R., Lim, W., Mutschler, M.A. and Earle, E.D. 2007. Complementary strategies for ploidy manipulations in gynogenic onion (*Allium cepa* L.). *Pl. Sci.,* **173**: 25-31

Anonymous. 2007. *NRCOG News*, Vol **11,** No. 2

Blakeslee, A.F., Belling, J., Farnham, M.E. and Bergner, A.D. 1922. A haploid mutant in the Jimson weed, *Datura stramonium*. *Sci.,* **55**:646-647

Bohancec, B. 2009. Doubled Haploids via Gynogenesis. In: Advances in Haploid Production in Higher Plants. Touraev, A., Forster, B.P. and Jain, S.M. (eds.). Springer-Science and Business Media B.V., The Netherlands, pp 35-46

Bohanec, B. 2002. Doubled haploid onions. p145-148. In: *Allium* crop science: Recent advances. Rabinowich, H. and Currah, L. (eds.), CABI Publishing House, Wallingford, UK

Bohanec, B. and Jakse, M. 1997.Characteristics of onion haploid induction procedure. In: *Procs. 1st Congr. Genet. Soc. Slovenia*, Ljubljana, Slovenia, pp. 47-49

Bohanec, B. and Jakse, M. 1999. Variations in gynogenic response among long-day onion (*Allium cepa* L.) accessions. *Pl. Cell Rep.,* **18**:737-742

Bohanec, B., Jakse, M. and Havey, M.J. 2003. Genetic analyses of gynogenetic haploid production in onion. *J. Amer. Soc. Hortl. Sci*., **128**:571-74

Bohanec, B., Jakse, M. and Javornik, B. 2001. Present status of haploid induction in onion: analysis of procedure and its limitations. In: *Procs. 2nd Int'l. Symp. Edible Alliaceae*. Armstrong, J. (ed.). *Acta Hort.*, **555:**91-98

Bohanec, B., Jakse, M., Ihan, A. and Javornik, B. 1995. Studies of gynogenesis in onion (*Allium cepa* L.): induction procedures and genetic analysis of regenerants. *Pl. Sci.,* **104**:215-224

Campion, B. and Alloni, C. 1990. Induction of haploid plants in onion (*Allium cepa* L.) by *in vitro* culture of unpollinated ovules. *Pl. Cell Tiss. Org. Cult.,* **20**:1-6

Campion, B., Azzimonti, M.T., Vicini, E., Schiayi, M. and Falavigna, A. 1992. Advances in haploid induction in onion (*Allium cepa* L.) through *in vitro* gynogenesis. *Pl. Sci.,* **86**:97-104

Campion, B., Perri, E., Azzimonti, M.T., Vicini, E. and Schiavi, M. 1995. Spontaneous and induced chromosome doubling in gynogenic lines of onion (*Allium cepa* L.). *Pl. Breed.,* **114**:243-46

Chen, J.F., Cui, L., Malik, A.A. and Mbira, K.G. 2010. *In vitro* haploid and dihaploid production *via* unfertilized ovule culture. *Pl.t Cell Tiss. Org. Cult.,* **68**:51-59

Cohat, J. 1994. *Obtention chez l'echalote* (*Allium cepa* L. var. *aggregatum*) *de plantes haploides gynogenetiques par culture in vitro de boutons floraux*. *Agronomie*,**14**:229-304

Dunstan, D.I. and Short, K.C. 1977. Improved growth of tissue cultures of onion (*Allium cepa*). *Physiol. Plant.,* **41**:70-72

Ebrahimi, R. and Zamani, Z. 2009. Effect of polyamines on *in vitro* gynogenesis of onion (*Allium cepa* L.). *Am-Eurasian J. Sustain. Agri.,* **3**:71-74

Forster, B.P., Heberle-Bors, E., Kasha, K.J. and Touraev, A. 2007. The resurgence of haploids in higher plants. *Trends Pl. Sci.,* **12:**368-375

Gamborg, O.C., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root

cells. *Exptl. Cell Res.,* **50**:151-58

Geoffriau, E., Kahane, R. and Rancillac, M. 1997. Variation of gynogenesis ability in onion (*Allium cepa* L.). *Euphytica,* **94**:37-44

Grzebelus, E. and Adamus, A. 2004. Effect of anti-mitotic agents on development and genome doubling of gynogenic onion (*Allium cepa* L.) embryos. *Pl. Sci.,***167**:569-574

Jakse, M., Bohanec, B. and Ihan, A. 1996. Effect of media components on the gynogenic regeneration of onion (*Allium cepa* L.) cultivars and analysis of regenerants. *Pl. Cell Rep.,* **15**:934-938

Jakse, M., Bohanec, B. and Havey, M.J. 2003. Chromosome doubling procedures of onion (*Allium cepa* L.) gynogenic embryos. *Pl. Cell Rep.,* **21**:905-910

Jakse, M., Hirschegger, P., Bohanec, B. and Havey, M.J. 2010. Evaluation of gynogenic responsiveness and pollen viability of selfed doubled haploid onion lines and chromosome doubling *via* somatic regeneration. *J. Amer. Soc. Hortl. Sci.,* **135**:67-73

Javornik, B., Bohanec, B. and Campion, B. 1998. Studies in the induction of a second cycle gynogenesis in onion (*Allium cepa* L.) and genetic analysis of the plant. *Pl. Breed.,* **117**:275-78

Keller, E.R.J. and Korzum, L. 1996. Haploidy in onion (*Allium cepa* L.) and other *Allium* species. In: *In vitro* haploid production in higher plants. Vol 3. Jain, S.M., Sopory, S.K. and Veilleux, R.E. (eds.). Springer Science and Business Media, B.V. Netherlands, pp. 51-75

Keller, J. 1990. Culture of unpollinated ovules, ovaries and flower buds in some species of the genus *Allium* and haploid induction via gynogenesis in onion (*Allium cepa* L.). *Euphytica,* **47**:241-247

Luthar, Z. and Bohanec, B. 1999. Induction of direct somatic organogenesis in onion (*Allium cepa* L.) using a two-step flower or ovary culture. *Pl. Cell Rep.,* **18**:797-802

Martinez, L.E., Augero, C.B., Lopez, M.E. and Galmarini, C.R. 2000. Improvement of *in vitro* gynogenesis induction in onion (*Allium cepa* L.) using polyamines. *Pl. Sci.,***156**:221-226

Michalik, B., Adamus, A. and Nowak, E. 2000. Gynogenesis in Polish onion cultivars. *J. Pl. Physiol.,* **156**:211-216

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.,* **15**:473-97

Muren, R.C. 1989. Haploid plant induction from unpollinated ovaries in onion. *Hort. Sci.,* **24**:833-34

Murovec, J. and Bohanec, B. 2012. Haploids and Doubled Haploids in Plant Breeding. In: Plant Breeding. Abdurakhmonov, I. (ed.). InTech, Europe, p87-106

Musial, K., Bohanec, B. and Przywara, L. 2001. Embryological study on gynogenesis in onion (*Allium cepa* L.). *Sex. Pl. Repr.,* **13**:335-41

Ponce, M., Martinez, L. and Galmarini, C. 2006. Influence of CCC, putrescine and gellan gum concentration on gynogenic embryo induction in *Allium cepa. Biol. Plant.,* **50**:425-428

Puddephat, I.J., Robinson, H.T., Smith, B.M. and Lynn, J. 1999. Influence of stock plant pre-treatment on gynogenic embryo induction from flower buds of onion. *Pl. Cell Tiss. Org. Cult.,* **57**:145-148

(MS Received 28 January 2014, Revised 01 May 2014, Accepted 17 June 2014)